

5

Nucleotide Sequences that Encode Phosphatidylinositol-3' Kinase
Associated Proteins and Uses Thereof

Inventor(s):

Sylvia Braselmann

Field of the Invention

5
10 The present invention is in the field of molecular biology, and particularly to cellular signal transduction involving nucleotide sequences that encode phosphatidylinositol-3'-kinase associated proteins, and uses of these proteins in the treatment and diagnosis of disease, preferably diseases caused by cell growth disorders.

15 **Background of the Invention**

Phosphatidylinositol-3'-kinases (PI3Ks) are a large family of enzymes capable of 3-phosphorylating at least one of the cellular phosphoinositides (Whitman et al., 1988, Nature 332:644-646; Auger et al., 1989, Cell 57:167-175). 3-phosphorylated phosphoinositides are found in all higher eukaryotic cells. A growing body of evidence implicates PI3K and a lipid product of this enzyme, phosphatidylinositol (3,4,5)-triphosphate (hereinafter "PtdIns(3,4,5)P₃"), as part of a novel and important second messenger system in cellular signal transduction. The components of this novel PtdIns(3,4,5)P₃-based signaling system appear to be independent of the previously characterized signaling pathway based on inositol phospholipids, in which a phosphoinositidase C (PIC) hydrolyses PtdIns(4,5)P₂ to release the structurally distinct second messengers inositol (1,4,5)-triphosphate (Ins(1,4,5)P₃) and diacylglycerol.

25
30 Select extracellular agonists and growth factors will stimulate intracellular PI3K activity and cause the rapid and transient intracellular accumulation of PtdIns(3,4,5)P₃. Surprisingly, stimulation of a variety of different types of cell surface receptors, including receptor tyrosine kinases, receptors associated with src family non-receptor tyrosine kinases, cytokine growth factors, and G protein coupled receptors will all activate members of the PI3K family. (Reviewed in Stephens et al., 1993, Biochemica et Biophysica Acta, 1179:27-75). For example, tyrosine kinase receptors which, when activated, result in increased accumulation of PtdIns(3,4,5)P₃ are the PDGF receptor, the EGF receptor, members of the FGF receptor family, the CSF-1 receptor, the insulin receptor,

the IGF-1 receptor, and the NGF receptor. Receptors associated with *src* family non-receptor tyrosine kinases which stimulate PtdIns(3,4,5)P₃ accumulation are the Il-2 receptor, Il-3 receptor, mlgM receptor, the CD4 receptor, the CD2 receptor, and the CD3/T cell receptor. Additionally, the cytokine Il-4 receptor and the G protein linked thrombin receptor, ATP receptor, and the fMLP receptor all stimulate the activity of a PI3K, resulting in subsequent PtdIns(3,4,5)P₃ accumulation. Thus, PtdIns(3,4,5)P₃ appears to be a second messenger in extremely diverse signaling pathways.

Support for the proposition that PI3K activity and production of PtdIns(3,4,5)P₃ is a physiological relevant pathway of signal transduction for these diverse receptors is derived, *inter alia*, from two different lines of experimental evidence: inhibition of PI3K activity by fungal metabolites and observations of direct protein associations. Wortmannin, a fungal metabolite, irreversibly inhibits PI3K activity by binding covalently to the catalytic domain of this enzyme. Inhibition of PI3K activity by Wortmannin eliminates the subsequent cellular response to the extracellular factor. For example, neutrophils respond to the chemokine fMet-Leu-Phe (fMLP) by stimulating PI3K and synthesizing PtdIns(3,4,5)P₃. The synthesis correlates with activation of the respiratory burst involved in neutrophil destruction of invading microorganisms. Treatment of neutrophils with Wortmannin prevents the fMLP-induced respiratory burst response. (Thelen et al., 1994, PNAS, USA 91:4960-4964.) Indeed, these experiments with Wortmannin, as well as other experimental evidence, shows that PI3K activity in cells of hematopoietic lineage, particularly neutrophils, monocytes, and other types of leukocytes, is involved in many of the non-memory immune responses associated with acute and chronic inflammation.

PI3K enzymes interact directly with, and may be co-purified with, activated forms of several receptor tyrosine kinases. When purified, receptor tyrosine kinase associated PI3K was found to consist of 170-200 kD heterodimers (Otsu et al., 1991, Cell 65:91-104, Pons et al., 1995, Mol. Cell. Biol. 15:4453-4465, Inukai et al., 1996, J. Biol. Chem. 271:5317-5320) comprising a catalytic subunit and an adapter (or regulatory) subunit.

Two different homologs of the catalytic subunit, p110 and p110 β , have been described and cloned. The catalytic subunit, which irreversibly binds Wortmannin, tightly associates with one or other members of a small family of highly related regulatory subunits, p55, p55PIK p85 α and p85 β , to form the 170-200 kD heterodimers. The known regulatory subunits contain a large collection

of protein:protein interaction domains, including two SH2 domains (Cantley et al., 1991, Cell 64:281-302).

The presence of the SH2 domains are thought to be responsible for the binding and stimulation of PI3K heterodimers to activated receptor tyrosine kinases. Activated receptors are phosphorylated at key tyrosine residues within local consensus sequences preferred by the SH2 domains found in the 55-87 kD PI3K adaptors (Songyang et al., 1993, Cell 72:767-778). Once the PI3K heterodimer binds, it directly activates the PI3K catalytic subunit (although this effect is relatively small *in vitro*, Carpenter et al., 1993, J. Biol. Chem. 268:9478-9483, Backer et al., 1992, EMBO J. 11:3469-3479) and translocates the cytosolic PI3K to a source of its phospholipid substrate. The combination of these factors leads to a surge in PtdIns(3,4,5)P₃ production. Clearly, these isoforms of PI3Ks seem structurally adapted to function as dedicated signal transducers downstream of receptor-regulated tyrosine kinases.

However, the p110/p85 sub-family of PI3Ks do not seem to be involved in the production of PtdIns(3,4,5)P₃ that can occur as a result of activation of cell surface receptors which utilize heterotrimeric GTPases to transduce their signals (e.g., fMLP, PAF, ATP, and thrombin). These types of cell surface receptors have been primarily described in cells of hematopoietic origin whose activation is involved in inflammatory responses of the immune system. Recent evidence has suggested that a chromatographically distinct form of Wortmannin-sensitive PI3K is present in U937 cells and neutrophils that possesses a native, relative molecular mass of about 220 kD (Stephens et al., 1994, Cell 77:83-93). This PI3K activity can be specifically stimulated by Gβγ subunits, but not Gα-GTP subunits. A similar PI3K activity has also been described in an osteosarcoma cell line (Morris et al., 1995, Mol. Pharm. 48:532-539). Platelets also contain a Gβγ-sensitive PI3K, although it is unclear whether this is a p85/p110 PI3K family member (Thomason et al., 1994, J. Biol. Chem. 269:16525-16528). It seemed likely that this poorly characterized, Gβγ sensitive PI3K might be responsible for production of PtdIns(3,4,5)P₃ in response to agonists like ATP, fMLP etc.

Identification of the mechanism by which PI3K activity is activated by cellular agonists which transduce their signals through G protein linked receptors is lacking. It is important to note that the majority of agonists which activate the neutrophil respiratory burst involved in the inflammatory response will bind to G-protein-coupled receptors rather than receptor tyrosine kinases. Thus, the mechanism by which PI3K is regulated in response to these types of chemokines

is likely to be different from regulation by growth factors which signal through tyrosine kinases.

Although there is some information concerning the initial events that affect the expression of PI3K, and the products resulting therefrom, much less is known concerning the factors that regulate the pathway from expression to product formation. It will be appreciated that the identification of such factors will have significant medical applications.

The present invention is directed towards resolving, at least in part, this issue by the identification, purification, and cloning of a novel PI3K associated protein.

SUMMARY OF THE INVENTION

A first object of the invention is the description of nucleotides that encode PI3K associated proteins.

A second object of the invention is the description of nucleotides that encode PI3K associated protein(s), which protein(s) bind to the intermediate SH2 domain on the regulatory subunit of p85 of PI3K, and further exhibit a bromodomain.

A third object of the invention is the description of nucleotides that encode PI3K associated protein(s), which protein(s) bind to the intermediate SH2 domain on the regulatory subunit of p85 of PI3K by the associated protein(s) C-terminal amino acids.

A fourth object of the invention is the description of methods for the expression of nucleotides that encode PI3K associated proteins, which proteins exhibit a bromodomain and the purification of the expressed proteins.

A fifth object of the invention is the description of isolated nucleic acid or protein fragments of PI3K associated proteins, respectively.

A sixth object of the invention is to describe host cells transformed with isolated nucleic acid sequences that encodes PI3K associated proteins or fragments thereof.

A seventh object of the invention is to describe vectors that contain isolated nucleic acid sequences that encode PI3K associated proteins or fragments thereof.

A eighth object of the invention is to describe complexes consisting of full length or fragments of PI3K associated proteins and PI3K.

A ninth object of the invention is to describe methods of diagnosing disease using isolated nucleic acid sequences, or fragments thereof, that encode PI3K associated proteins, or fragments thereof.

A tenth object of the invention is to describe an assay for identifying compounds that would have prophylactic or therapeutic applications for the treatment of disease, preferably agonists and antagonists of PI3K regulation,

including small molecules, mutant PI3K associated proteins that compete with native PI3K associated proteins for binding to the regulatory subunit of PI3K, antibodies, and nucleotide sequences that can be used to inhibit PI3K associated protein gene expression (e.g., antisense and ribozyme molecules, and gene or regulatory sequence replacement constructs) or to enhance PI3K associated protein gene expression (e.g., expression constructs that place the PI3K associated protein gene under the control of a strong promoter system), and transgenic cells and animals that express a PI3K associated protein transgene or "knock-outs" that do not express PI3K associated protein. Such diseases would include those involving unregulated cell growth, including restinosis, and cancer.

These and other objects of the present invention will become apparent to one of ordinary skill in the art upon reading the description of the various aspects of the invention in the following specification. The foregoing and other aspects of the present invention are explained in greater detail in the drawings, detailed description, and examples set forth below.

DEFINITIONS

As used herein, the following terms, whether used in the singular or plural, will have the meanings indicated:

G protein-regulated PI3K: refers to a PI3K enzyme whose activity is stimulated by activated trimeric G proteins such as $G\beta\gamma$ subunits and/or $G\alpha$ -GTP subunits.

~~p85: means the regulatory subunit of PI3K, also known as the adapter subunit. p85 includes molecules that are homologous to p85 or which bind to p110, tyrosine kinase receptor-regulated PI3K, at the inter SH2 domain (the region between the two SH2 domains and consisting of amino acids 434 to 599, according to the amino acid position numbering of Klippel et al., Mol. Cell. Biol., vol. 13: 5560 (1993)), and stimulate PI3K catalytic activity.~~

p85 nucleotides or coding sequences: means nucleotide sequences encoding the p85 regulatory subunit protein, polypeptide or peptide fragments of p85, or p85 fusion proteins. p85 nucleotide sequences encompass DNA, including genomic DNA (e.g. the p85 gene) or cDNA, or RNA. Human p85 has been cloned as described by Skolnik et al., Cell, vol. 65 : pages 83-90 (1991)

p110: means the catalytic subunit of PI3K. Functional equivalents of p110 refer to a PI3K catalytic subunit protein which binds to the p85 regulatory subunit with high affinity *in vivo* or *in vitro*. Other functional equivalents of p110 are homologous catalytic subunits of PI3K.

p110 nucleotides or coding sequences: means nucleotide sequences encoding the p110 catalytic subunit protein, polypeptide or peptide fragments of p110 protein, or p110 fusion proteins. p110 nucleotide sequences encompass DNA, including genomic DNA (e.g. the p110 gene) or cDNA, or RNA. Human p110 has been cloned as described by Hu et al. Mol. Cell. Biol., vol. 13 (12) pages 7677-7688

DESCRIPTION OF THE TABLES

Table 1 shows the plasmid constructs containing certain regions of PIKAP and p85 that were used to perform two-hybrid assays.

Table 2 shows the two-hybrid screening results with specific constructs of PIKAP and p85 which establish that binding of PIKAP to p85 is to the inter SH2 domain of p85, and through the c-terminal end of PIKAP.

DETAILED DESCRIPTION OF THE INVENTION

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The present invention relates to the identification, purification, and cloning of PI3K associated protein(s) which protein(s) bind to the inter SH2 domain (the region between the two SH2 domains) on the regulatory subunit of p85 of PI3K by the associated protein(s) C-terminal amino acids, and preferably through no more than the last 74 C-terminal amino acids. Such proteins also exhibit a bromodomain.

PI3K are enzymes which phosphorylate phosphatidyl inositols at the 3d position to generate the intracellular signaling molecule PtdIns(3,4,5)P₃. It has been shown that PI3K's are induced in a variety of cell types upon stimulation of tyrosine kinase receptors.

Reported herein is the discovery that certain proteins associate with PI3K and affect its activity. Thus, the invention encompasses such proteins, termed PI3K Associated Proteins (PIKAP), or peptides, the use of PIKAP nucleotides, as well as antibodies to PIKAP which can, for example, act as PIKAP agonists or antagonists, antagonists that inhibit PI3K activity or expression, or agonists that activate PI3K activity in the treatment of PI3K cell activation disorders, including, but not limited to diseases associated with uncontrolled cell growth (cell growth disorders), preferably restinosis and cancer.

Further, the invention encompasses the use of PI3K Associated Protein (PIKAP) nucleotides, proteins and peptides, as well as antibodies to PIKAP in the diagnosis of PI3K cell activation disorders. The diagnosis of an abnormality PI3K Associated Protein (PIKAP) nucleotides, proteins or peptides in a patient, or an abnormality in the activated PI3K signal transduction pathway, will also assist in devising a proper treatment or therapeutic regimen.

In particular, the invention described in the sections below encompasses PIKAP, polypeptides or peptides that display functional domains that interact with the regulatory subunit of PI3K, preferably p85, or the same that is mutated, truncated or deleted (e.g. a regulatory subunit of PI3K or a functional domain of the regulatory subunit, fused to an unrelated protein or peptide such as an epitope tag, i.e., the myc epitope), nucleotide sequences encoding such products, and host cell expression systems that can produce such regulatory subunit products.

The invention also encompasses antibodies and anti-idiotypic antibodies (including Fab fragments), antagonists and agonists of PIKAP, as well as compounds or nucleotide constructs that inhibit expression of nucleotide sequences that encode PIKAP (transcription factor inhibitors, antisense and ribozyme molecules, or gene or regulatory sequence replacement constructs), or promote expression of PIKAP (e.g., expression constructs in which PIKAP coding sequences are operatively associated with expression control elements such as promoters, promoter/enhancers, etc.). The invention also relates to host cells and animals genetically engineered to express the human PIKAP (or mutants thereof) or to inhibit or "knock-out" expression of the animal's endogenous PIKAP.

PIKAP, peptides, fusion proteins, nucleotide sequences, antibodies, antagonists and agonists can be useful for the detection of mutant PIKAP or inappropriately expressed PIKAP for the diagnosis of disease, preferably immune disorders. The above compositions alone or in combination with host cell expression systems, antibodies, antagonists, agonists and genetically engineered cells and animals can be used for screening for drugs effective in the treatment of such disease. The use of engineered host cells and/or animals may offer an advantage in that such systems allow not only for the identification of compounds that bind to the PIKAP, but can also identify compounds that affect the signal transduced by the PI3K, specifically, production of the intracellular signaling molecule $\text{PtdIns}(3,4,5)\text{P}_3$.

Finally, PIKAP, fusion protein products, antibodies and anti-idiotypic antibodies (including Fab fragments), antagonists or agonists (including

compounds that modulate signal transduction which may act on downstream targets in the PI3K signal transduction pathway can be used for therapy of such diseases. For example, nucleotide constructs encoding functional PIKAP, mutant PIKAP, as well as antisense and ribozyme molecules can be used in "gene therapy" approaches for the modulation of PIKAP expression and/or activity in the treatment of disease.

Numerous methods are available for isolating nucleotide sequences that encodes PIKAP, and preferably a cDNA sequence. One method takes advantage of the association of PIKAP with PI3K, and is generally used to identify protein-protein interactions. It is the two-hybrid system, and it is described herein in detail for illustration only and not by way of limitation. This system has been described in U. S. Patent No. 5, 283, 173 and by Chien et al., 1991, Proc. Natl. Acad. Sci. USA, 88:9578-9582, and is commercially available from Clontech (Palo Alto, CA). Briefly, utilizing such a system, plasmids are constructed that encode two hybrid proteins: one plasmid consists of nucleotides encoding the DNA-binding domain of a transcription activator protein fused to a PI3K p85 nucleotide sequence encoding p85, or p85 peptide or fusion protein, and the other plasmid consists of nucleotides encoding the transcription activator protein's activation domain fused to a cDNA encoding an unknown protein, or in the instant case PIKAP, which has been recombined into this plasmid as a part of the cDNA library. The DNA-binding domain fusion plasmid and the cDNA library are transformed into a strain of yeast Saccharomyces cerevisiae that contains a reporter gene (e.g., HIS3 or lacZ) with a regulatory region that contains the transcription activator's binding site. Either hybrid protein alone cannot activate transcription of the reporter gene; the DNA-binding domain hybrid cannot because it does not provide activation function, and the activation domain hybrid cannot because it cannot localize to the activator's binding sites. Interaction of the two hybrid proteins reconstitutes the functional activator protein and results in transcriptional activation of the reporter gene, which is detected by an assay for the reporter gene product.

The two-hybrid system or related methodology may be used to screen activation domain libraries for proteins that interact with the "bait" gene product. By way of example, and not by way of limitation, preferably p85 is used as the bait gene product. Total genomic or cDNA sequences are fused to the DNA encoding an activation domain. This library and the plasmid encoding a hybrid of a bait p85 gene product fused to the DNA-binding domain are cotransformed into a yeast reporter strain, and the resulting transformants are screened for those that have transcriptionally activated reporter gene. For example, and not by way of limitation, a bait p85 gene sequence can be cloned into a vector such that it is

translationally fused to the DNA encoding the DNA-binding domain of the GAL4 protein. These colonies are purified and the library plasmids responsible for reporter gene transcription are isolated. DNA sequencing is then used to determine the nucleotide sequence of the clones which, in turn, reveals the identity of the protein sequences encoded by the library plasmids.

A cDNA library of the cell line from which proteins that interact with bait p85 gene product are to be detected can be made using methods routinely practiced in the art. According to the particular system described herein, for example, the cDNA fragments can be inserted into a vector such that they are translationally fused to the transcriptional activation domain of GAL4. This library can be co-transformed along with the bait p85 gene-GAL4 fusion plasmid into a yeast strain which contains a lacZ gene driven by a promoter which contains GAL4 activation sequence. A cDNA encoded protein, fused to GAL4 transcriptional activation domain, that interacts with bait p85 gene product will reconstitute an active GAL4 protein and thereby drive expression off the HIS3 gene. Colonies which express HIS3 can be detected by their growth on petri dishes containing semi-solid agar based media lacking histidine. The cDNA can then be purified from these strains, and used to produce and isolate the p85 gene-interacting protein using techniques routinely practiced in the art.

Using the above described two-hybrid technique a PI3 Kinase associated protein (PIKAP) was identified, and shown to have certain unique properties, including a bromodomain. Briefly, the cDNA encoding human PI3K p85 (see Skolnik et al., Cell 65:83-90) was digested with Asp 718 and PstI and the full length p85 sequence was fused to the GAL4 binding domain in the SmaI-PstI sites of pGBT8 plasmid, which is the pMA424 plasmid of Chien et al. as described in Proc. Natl. Acad. Sci. vol. 88: pages 9578-9582 (1991), modified by the insertion of the sequence 5'-~~C~~GGGGATCCCCATGGCTAGCCATATG-3' between the EcoRI and Sall unique sites. This was transformed into the yeast strain YGH1, and the YGH1 strain carrying the plasmid GAL4-p85 was evaluated for its intrinsic ability to activate the two reporters-growth in histidine minus media and β -galactosidase activity. The YGH1 strain carrying the plasmid GAL4-p85 was subsequently transformed with a HeLa cell cDNA library fused to the GAL4 activation domain in the pGAD plasmid (Chien et al., Proc. Natl. Acad. Sci. vol. 88: pages 9578-9582 (1991). When a cDNA encodes a PIKAP that interacts with the p85, the YGH1 strain is expected to grow in the absence of histidine and produce β -galactosidase.

One screening was done with full length p85 fused to Gal 4 DNA binding domain (Asp 718-PstI, amino acids (AA) 6 to the end of p85). 2×10^5

transformants were screened. 14 clones conferred ability to grow in the absence of histidine and to produce β -galactosidase. The plasmids recovered from these yeast strains were used to re-transform the original YGH1 GAL4-p85 strain.

Again, all the plasmids conferred the ability to grow in the absence of histidine

5 and to produce β -galactosidase. Upon subsequent screening, 12 of the 14 were found to have cDNAs that encode PIKAP that clearly bound to p85. The cDNAs encode various NH2 terminal truncations; the largest had 1232 base pairs and exhibited an open reading frame of 943 bp. Northern blot analysis on human tissue mRNAs using a radio labeled PCR probe corresponding to bp 1493-1683
10 of the full length sequence revealed a mRNA of about 2.4 Kb.

Thus, the missing 5' sequences of PIKAP were isolated from a human pancreatic cDNA library (Stratagene, Corp.) using sequences present in PIKAP, and these are bp 1160-1460. A clone was isolated containing 2307 base pairs plus a polyadenosine tail of 20 adenosines. It exhibits a start codon at base pair
15 162, and an open reading frame of 1767 base pairs, and thus encodes a protein with 589 amino acids.

The cDNA sequence (SEQ. ID. No. 1) and deduced amino acid sequence (SEQ. ID. No. 2) of human PIKAP are shown in the Sequence Listing, respectively. The calculated molecular weight is 69 kDa. Particularly noteworthy
20 is the presence of a bromodomain.

The nucleotide sequence encoding the last 74 amino acids of PIKAP is believed to be sufficient to bind p85. Other domains of PIKAP are described by amino acid residues from about 151 to 313 define a bromodomain (Haynes et al., Nucleic Acids Research, Vol. 20, No. 10, p2603) Bromodomains may be involved
25 in protein protein interactions.

The PIKAP nucleotide sequences of the invention include: (a)the DNA sequence shown in FIG. 1 or contained in the cDNA clone PIKAP as deposited with the American Type Culture Collection (ATCC) under accession number 98189; (b)nucleotide sequence that encodes the amino acid sequence shown in
30 FIG. 2, or the PIKAP amino acid sequence encoded by the cDNA clone PIKAP as deposited with the ATCC; (c)any nucleotide sequence that hybridizes to the complement of the DNA sequence shown in FIG. 1 or contained in the cDNA clone pPIKAP as deposited with the ATCC under highly stringent conditions, e.g., hybridization to filter-bound DNA in 0.5 M NaHPO₄, 7% sodium dodecyl sulfate
35 (SDS), 1mM EDTA at 65%C, and washing in 0.1xSSC/0.1% SDS at 68%C (Ausubel F.M. et al., eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York, at p. 2.10.3) and encodes a functionally equivalent gene product; and (d) any

nucleotide sequence that hybridizes to the complement of the DNA sequences that encode the amino acid sequence shown in FIG. 1 or contained in the cDNA clone of the pPIKAP as deposited with the ATCC under less stringent conditions, such as moderately stringent conditions, e.g., washing in 0.2xSSC/0.1% SDS at 42°C (Ausubel et al., 1989, supra), yet which still encodes a functionally equivalent PIKAP gene product. Functional equivalents of the PIKAP include naturally occurring PIKAP present in other species, and mutant PIKAPs whether naturally occurring or engineered which retain at least some of the functional activities of PIKAP (i.e., binding to the p85. The invention also includes degenerate variants of sequences (a) through (d).

The invention also includes nucleic acid molecules, preferably DNA molecules, that hybridize to, and are therefore the complements of, the nucleotide sequences (a) through (d), in the preceding paragraph. Such hybridization conditions may be highly stringent or less highly stringent, as described above. In instances wherein the nucleic acid molecules are deoxyoligonucleotides ("oligos"), highly stringent conditions may refer, e.g., to washing in 6xSSC/0.05% sodium pyrophosphate at 37°C (for 14-base oligos), 48°C (for 17-base oligos), 55°C (for 20-base oligos), and 60°C (for 23-base oligos). These nucleic acid molecules may encode or act as PIKAP antisense molecules, useful, for example, in PIKAP gene regulation (for and/or as antisense primers in amplification reactions of PIKAP gene nucleic acid sequences). With respect to PIKAP gene regulation, such techniques can be used to regulate, for example, inflammatory immune responses. Further, such sequences may be used as part of ribozyme and/or triple helix sequences, also useful for PIKAP gene regulation. Still further, such molecules may be used as components of diagnostic methods whereby, for example, the presence of a particular PIKAP allele responsible for causing an inflammatory response, such as arthritis, may be detected.

In addition to the PIKAP nucleotide sequences described above, full length PIKAP cDNA or gene sequences present in the same species and/or homologs of the PIKAP gene present in other species can be identified and readily isolated, without undue experimentation, by molecular biological techniques well known in the art. Therefore, homologs of PIKAP can be readily identified and isolated from a cDNA library.

The identification of homologs of PIKAP in related species can be useful for developing animal model systems more closely related to humans for purposes of drug discovery. For example, expression libraries of cDNAs synthesized from neutrophil mRNA derived from the organism of interest can be screened using labeled catalytic subunit derived from that species, e.g., a p110, or p110

catalytic subunit fusion protein. Alternatively, such cDNA libraries, or genomic DNA libraries derived from the organism of interest can be screened by hybridization using the nucleotides described herein as hybridization or amplification probes. Furthermore, genes at other genetic loci within the genome that encode proteins which have extensive homology to one or more domains of the PIKAP gene product can also be identified via similar techniques. In the case of cDNA libraries, such screening techniques can identify clones derived from alternatively spliced transcripts in the same or different species.

Screening can be by filter hybridization, using duplicate filters. The labeled probe can contain at least 15-30 base pairs of the PIKAP nucleotide sequence, as shown in FIG. 1. The hybridization washing conditions used should be of a lower stringency when the cDNA library is derived from an organism different from the type of organism from which the labeled sequence was derived.

Low stringency conditions are well known to those of skill in the art, and will vary predictably depending on the specific organisms from which the library and the labeled sequences are derived. For guidance regarding such conditions see, for example, Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, Cold Springs Harbor Press, N.Y.; and Ausubel et al., 1989, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y.

Alternatively, the labeled PIKAP nucleotide probe may be used to screen a genomic library derived from the organism of interest, again, using appropriately stringent conditions. The identification and characterization of human genomic clones is helpful for designing diagnostic tests and clinical protocols for treating cell growth disorders in human patients. For example, sequences derived from regions adjacent to the intron/exon boundaries of the human gene can be used to design primers for use in amplification assays to detect mutations within the exons, introns, splice sites (e.g. splice acceptor and/or donor sites), etc., that can be used in diagnostics.

Further, a PIKAP gene homolog may be isolated from nucleic acid of the organism of interest by performing PCR using two degenerate oligonucleotide primer pools designed on the basis of amino acid sequences within the PIKAP gene product disclosed herein. The template for the reaction may be cDNA obtained by reverse transcription of mRNA prepared from, for example, human or non-human cell lines or cell types, such as neutrophils, known or suspected to express a PIKAP gene allele.

The PCR product may be subcloned and sequenced to ensure that the amplified sequences represent the sequences of a PIKAP gene. The PCR fragment may then be used to isolate a full length cDNA clone by a variety of methods. For example, the amplified fragment may be labeled and used to

screen a cDNA library, such as a bacteriophage cDNA library. Alternatively, the labeled fragment may be used to isolate genomic clones via the screening of a genomic library.

PCR technology may also be utilized to isolate full length cDNA sequences. For example, RNA may be isolated, following standard procedures, from an appropriate cellular source (i.e., one known, or suspected, to express the PIKAP gene, such as, for example, neutrophils or other types of leukocytes). A reverse transcription reaction may be performed on the RNA using an oligonucleotide primer specific for the most 5' end of the amplified fragment for the priming of first strand synthesis. The resulting RNA/DNA hybrid may then be "tailed" with guanines using a standard terminal transferase reaction, the hybrid may be digested with RNAase H, and second strand synthesis may then be primed with a poly-C primer. Thus, cDNA sequences upstream of the amplified fragment may easily be isolated. For a review of cloning strategies which may be used, see e.g., Sambrook et al., 1989, supra.

The PIKAP gene sequences may additionally be used to isolate mutant PIKAP gene alleles. Such mutant alleles may be isolated from individuals either known or proposed to have a genotype which contributes to the symptoms of hematopoietic lineage cell activation disorders such as inflammation. Mutant alleles and mutant allele products may then be utilized in the therapeutic and diagnostic systems described below. Additionally, such PIKAP gene sequences can be used to detect PIKAP gene regulatory (e.g., promoter or promotor/enhancer) defects.

A cDNA of a mutant PIKAP gene may be isolated, for example, by using PCR, a technique which is well known to those of skill in the art. In this case, the first cDNA strand may be synthesized by hybridizing an oligo-dT oligonucleotide to mRNA isolated from cells known or suspected to be expressed in an individual putatively carrying the mutant PIKAP allele, and by extending the new strand with reverse transcriptase. The second strand of the cDNA is then synthesized using an oligonucleotide that hybridizes specifically to the 5' end of the normal gene. Using these two primers, the product is then amplified via PCR, cloned into a suitable vector, and subjected to DNA sequence analysis through methods well known to those of skill in the art. By comparing the DNA sequence of the mutant PIKAP allele to that of the normal PIKAP allele, the mutation(s) responsible for the loss or alteration of function of the mutant PIKAP gene product can be ascertained.

Alternatively, a genomic library can be constructed using DNA obtained from an individual suspected of or known to carry the mutant PIKAP allele, or a cDNA library can be constructed using RNA from a cell type known, or suspected, to

express the mutant PIKAP allele. The normal PIKAP gene or any suitable fragment thereof may then be labeled and used as a probe to identify the corresponding mutant PIKAP allele in such libraries. Clones containing the mutant PIKAP gene sequences may then be purified and subjected to sequence analysis according to methods well known to those of skill in the art.

Additionally, an expression library can be constructed utilizing cDNA synthesized from, for example, RNA isolated from a cell type known, or suspected, to express a mutant PIKAP allele in an individual suspected of or known to carry such a mutant allele. In this manner, gene products made by the putatively mutant cell type may be expressed and screened using standard antibody screening techniques in conjunction with antibodies raised against the normal PIKAP gene product, as described, below (For screening techniques, see, for example, Harlow, E. and Lane, eds., 1988, "Antibodies: A Laboratory Manual", Cold Spring Harbor Press, Cold Spring Harbor.) Additionally, screening can be accomplished by screening with labeled fusion proteins. In cases where a PIKAP mutation results in an expressed gene product with altered function (e.g., as a result of a missense or a frameshift mutation), a polyclonal set of antibodies to PIKAP are likely to cross-react with the mutant PIKAP gene product. Library clones detected via their reaction with such labeled antibodies can be purified and subjected to sequence analysis according to methods well known to those of skill in the art.

The invention also encompasses nucleotide sequences that encode mutant PIKAPs, peptide fragments of the PIKAP, truncated PIKAPs, and PIKAP fusion proteins. These include, but are not limited to nucleotide sequences encoding mutant PIKAPs described below; polypeptides or peptides corresponding to the catalytic binding, or G β subunit binding domains of the PIKAP or portions of these domains; truncated PIKAPs in which one or two of the domains is deleted, or a truncated, nonfunctional PIKAP. Nucleotides encoding fusion proteins may include but are not limited to full length PIKAP, truncated PIKAP or peptide fragments of PIKAP fused to an unrelated protein or peptide, such as for example, an epitope tag which aids in purification or detection of the resulting fusion protein; or an enzyme, fluorescent protein, luminescent protein which can be used as a marker.

The invention also encompasses (a) DNA vectors that contain any of the foregoing PIKAP coding sequences and/or their complements (i.e., antisense); (b) DNA expression vectors that contain any of the foregoing PIKAP coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences; and (c) genetically engineered host cells that contain any of the foregoing PIKAP coding sequences operatively

associated with a regulatory element that directs the expression of the coding sequences in the host cell. As used herein, regulatory elements include but are not limited to inducible and non-inducible promoters, enhancers, operators and other elements known to those skilled in the art that drive and regulate
5 expression. Such regulatory elements include but are not limited to the baculovirus promoter, cytomegalovirus hCMV immediate early gene, the early or late promoters of SV40 adenovirus, the lac system, the trp system, the TAC system, the TRC system, the major operator and promoter regions of phage A, the control regions of fd coat protein, the promoter for 3-phosphoglycerate
10 kinase, the promoters of acid phosphatase, and the promoters of the yeast - mating factors.

PIKAP

PIKAP can be prepared for a variety of uses, including but not limited to the
15 generation of antibodies, as reagents in diagnostic assays, the identification of other cellular gene products involved in the regulation of cell growth disorders, and as reagents in assays for screening for compounds that can be used in the treatment of such disorders.

The PIKAP amino acid sequences of the invention include the amino acid
20 sequence shown in FIG. 2 (SEQ. ID. No:2), or the amino acid sequence encoded by the cDNA clone PIKAP, as deposited with the ATCC. Further, PIKAPs of other species are encompassed by the invention. In fact, any PIKAP protein encoded by the PIKAP nucleotide sequences described above, are within the scope of the invention.

25 The invention also encompasses proteins that are functionally equivalent to the PIKAP encoded by the nucleotide sequences described above, as judged by any of a number of criteria, including but not limited to the ability to bind p85, the ability to affect PI3K activity. Such functionally equivalent PIKAP proteins include but are not limited to additions or substitutions of amino acid residues within the
30 amino acid sequence encoded by the PIKAP nucleotide sequences described, above, but which result in a silent change, thus producing a functionally equivalent gene product. Amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, nonpolar
35 (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

While random mutations can be made to PIKAP DNA (using random mutagenesis techniques well known to those skilled in the art) and the resulting mutant PIKAPs tested for activity, site-directed mutations of the PIKAP coding sequence can be engineered (using site-directed mutagenesis techniques well known to those skilled in the art) to generate mutant PIKAPs with increased function, e.g., higher binding affinity for catalytic subunit, and/or greater signaling capacity; or decreased function, e.g., lower binding affinity for catalytic subunit, and/or decreased signal transduction capacity.

For example, porcine PIKAP amino acid sequence may be aligned with that of human PIKAP. Mutant PIKAPs can be engineered so that regions of interspecies identity are maintained, whereas the variable residues are altered, e.g., by deletion or insertion of an amino acid residue(s) or by substitution of one or more different amino acid residues. Conservative alterations at the variable positions can be engineered in order to produce a mutant PIKAP that retains function. Non-conservative changes can be engineered at these variable positions to alter function. Alternatively, where alteration of function is desired, deletion or non-conservative alterations of the conserved regions can be engineered. One of skill in the art may easily test such mutant or deleted PIKAPs for these alterations in function using the teachings presented herein.

Other mutations to the PIKAP coding sequence can be made to generate PIKAPs that are better suited for expression, scale up, etc. in the host cells chosen. For example, the triplet code for each amino acid can be modified to conform more closely to the preferential codon usage of the host cell's translational machinery.

Peptides corresponding to one or more domains (or a portion of a domain) of PIKAP (e.g., the p85 binding, bromodomain), truncated or deleted PIKAPs (e.g., PIKAP in which portions of one or more of the above domains are deleted) as well as fusion proteins in which the full length PIKAP, a PIKAP peptide or truncated PIKAP is fused to an unrelated protein are also within the scope of the invention and can be designed on the basis of the PIKAP nucleotide and PIKAP amino acid sequences disclosed in this section and above. Such fusion proteins include but are not limited to fusions to an epitope tag (such as is exemplified in the Examples below); or fusions to an enzyme, fluorescent protein, or luminescent protein which provide a marker function.

While the PIKAP polypeptides and peptides can be chemically synthesized (e.g., see Creighton, 1983, *Proteins: Structures and Molecular Principles*, W.H. Freeman & Co., N.Y.), large polypeptides derived from the PIKAP and the full length PIKAP itself may advantageously be produced by recombinant DNA technology using techniques well known in the art for expressing nucleic acid

containing PIKAP gene sequences and/or coding sequences. Such methods can be used to construct expression vectors containing the PIKAP nucleotide sequences described above and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. See, for example, the techniques described in Sambrook et al., 1989, supra, and Ausubel et al., 1989, supra. Alternatively, RNA capable of encoding PIKAP nucleotide sequences may be chemically synthesized using, for example, synthesizers. See, for example, the techniques described in "Oligonucleotide Synthesis", 1984, Gait, M.J. ed., IRL Press, Oxford, which is incorporated by reference herein in its entirety.

A variety of host-expression vector systems may be utilized to express the PIKAP nucleotide sequences of the invention. Where the PIKAP peptide or polypeptide is a soluble derivative the peptide or polypeptide can be recovered from the culture, i.e., from the host cell in cases where the PIKAP peptide or polypeptide is not secreted, and from the culture media in cases where the PIKAP peptide or polypeptide is secreted by the cells. However, such engineered host cells themselves may be used in situations where it is important not only to retain the structural and functional characteristics of the PIKAP, but to assess biological activity, e.g., in drug screening assays.

The expression systems that may be used for purposes of the invention include but are not limited to microorganisms such as bacteria (e.g., E. coli, B. subtilis) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing PIKAP nucleotide sequences; yeast (e.g., Saccharomyces, Pichia) transformed with recombinant yeast expression vectors containing the PIKAP nucleotide sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing the PIKAP sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing PIKAP nucleotide sequences; or mammalian cell systems (e.g., COS, CHO, BHK, 293, 3T3, U937) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the PIKAP gene product being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions of PIKAP protein or

for raising antibodies to the PIKAP protein, for example, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the E. coli expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:1791), in which the PIKAP coding sequence may be ligated individually into the vector in frame with the lacZ coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic Acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 264:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). If the inserted sequence encodes a relatively small polypeptide (less than 25 kD), such fusion proteins are generally soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety. Alternatively, if the resulting fusion protein is insoluble and forms inclusion bodies in the host cell, the inclusion bodies may be purified and the recombinant protein solubilized using techniques well known to one of skill in the art.

In an insect system, Autographa californica nuclear polyhidrosis virus (AcNPV) may be used as a vector to express foreign genes. (E.g., see Smith et al., 1983, J. Virol. 46: 584; Smith, U.S. Patent No. 4,215,051).

In mammalian host cells, a number of viral-based expression systems may be utilized preferably using a CMV promoter to transiently express recombinant protein in U937 cells or in Cos-7 cells. Alternatively, retroviral vector systems well known in the art may be used to insert the recombinant expression construct into host cells. For example, retroviral vector systems for transducing cells are described in published PCT applications WO 96/09400 and WO 94/29438.

In cases where an adenovirus is used as an expression vector, the PIKAP nucleotide sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the PIKAP gene product in infected hosts. (E.g., See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 81:3655-3659). Specific initiation signals may also be required for efficient translation of inserted PIKAP nucleotide sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where an entire PIKAP gene or cDNA, including its own initiation codon and adjacent sequences, is inserted into the

appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of the PIKAP coding sequence is inserted, exogenous translational control signals, including, perhaps, the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (See Bittner et al., 1987, Methods in Enzymol. 153:516-544).

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript may be used. Such mammalian host cells include but are not limited to CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, and U937 cells.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the PIKAP sequences described above may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the PIKAP gene product. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that affect the endogenous activity of the PIKAP gene product.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler et al., 1977, Cell 11:223),

hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy et al., 1980, Cell 22:817) genes can be employed in tk⁻, hgprt⁻ or aprt⁻ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin et al., 1981, J. Mol. Biol. 150:1); and hygromycin (Santerre et al., 1984, Gene 30:147).

The PIKAP gene product can also be expressed in transgenic animals. Animals of any species, including, but not limited to, mice, rats, rabbits, guinea pigs, pigs, micro-pigs, goats, and non-human primates, e.g., baboons, monkeys, and chimpanzees may be used to generate PIKAP transgenic animals.

Any technique known in the art may be used to introduce the PIKAP transgene into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to pronuclear microinjection (Hoppe, P.C. and Wagner, T.E., 1989, U.S. Pat. No. 4,873,191); retrovirus mediated gene transfer into germ lines (Van der Putten et al., 1985, Proc. Natl. Acad. Sci., USA 82:6148-6152); gene targeting in embryonic stem cells (Thompson et al., 1989, Cell 56:313-321); electroporation of embryos (Lo, 1983, Mol Cell. Biol. 3:1803-1814); and sperm-mediated gene transfer (Lavitrano et al., 1989, Cell 57:717-723); etc. For a review of such techniques, see Gordon, 1989, Transgenic Animals, Intl. Rev. Cytol. 115:171-229, which is incorporated by reference herein in its entirety.

The present invention provides for transgenic animals that carry the PIKAP transgene in all their cells, as well as animals which carry the transgene in some, but not all their cells, i.e., mosaic animals. The transgene may be integrated as a single transgene or in concatamers, e.g., head-to-head tandems or head-to-tail tandems. The transgene may also be selectively introduced into and activated in a particular cell type by following, for example, the teaching of Lasko et al. (Lasko, M. et al., 1992, Proc. Natl. Acad. Sci. USA 89: 6232-6236). The regulatory sequences required for such a cell-type specific activation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art. When it is desired that the PIKAP gene transgene be integrated into the chromosomal site of the endogenous PIKAP gene, gene targeting is preferred. Briefly, when such a technique is to be utilized, vectors containing some

nucleotide sequences homologous to the endogenous PIKAP gene are designed for the purpose of integrating, via homologous recombination with chromosomal sequences, into and disrupting the function of the nucleotide sequence of the endogenous PIKAP gene. In this way, the expression of the endogenous PIKAP gene may also be eliminated by inserting non-functional sequences into the endogenous gene. The transgene may also be selectively introduced into a particular cell type, thus inactivating the endogenous PIKAP gene in only that cell type, by following, for example, the teaching of Gu et al. (Gu et al., 1994, Science 265: 103-106). The regulatory sequences required for such a cell-type specific inactivation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art.

Once transgenic animals have been generated, the expression of the recombinant PIKAP gene may be assayed utilizing standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques to analyze animal tissues to assay whether integration of the transgene has taken place. The level of mRNA expression of the transgene in the tissues of the transgenic animals may also be assessed using techniques which include but are not limited to Northern blot analysis of cell type samples obtained from the animal, *in situ* hybridization analysis, and RT-PCR. Samples of PIKAP gene-expressing tissue, may also be evaluated immunocytochemically using antibodies specific for the PIKAP transgene product, as described below.

DIAGNOSIS OF CELL GROWTH DISORDERS

A variety of methods can be employed for the diagnostic and prognostic evaluation of cell growth disorders, including restinosis and cancer, and for the identification of subjects having a predisposition to such disorders.

Such methods may, for example, utilize reagents such as the PIKAP nucleotide sequences, and PIKAP antibodies, as described above. Specifically, such reagents may be used, for example, for: (1) the detection of the presence of PIKAP gene mutations, or the detection of either over- or under-expression of PIKAP mRNA relative to the non cell growth disorder state; (2) the detection of either an over- or an under-abundance of PIKAP gene product relative to the non-cell growth disorder state; and (3) the detection of perturbations or abnormalities in the signal transduction pathway mediated by PIKAP.

The methods described herein may be performed, for example, by utilizing pre-packaged diagnostic kits comprising at least one specific PIKAP nucleotide sequence or PIKAP antibody reagent described herein, which may be conveniently used, e.g., in clinical settings, to diagnose patients exhibiting cell activation disorder abnormalities.

For the detection of PIKAP, any nucleated cell can be used as a starting source for genomic nucleic acid. For the detection of PIKAP expression, any cell type or tissue in which the PIKAP is expressed may be utilized.

Nucleic acid-based detection techniques are described, below. Peptide
5 detection techniques are also described below.

DETECTION OF THE PIKAP GENE AND TRANSCRIPTS

Mutations within the PIKAP gene(s) can be detected by utilizing a number of techniques. Nucleic acid from any nucleated cell can be used as the starting
10 point for such assay techniques, and may be isolated according to standard nucleic acid preparation procedures which are well known to those of skill in the art.

DNA may be used in hybridization or amplification assays of biological samples to detect abnormalities involving gene structure, including point
15 mutations, insertions, deletions and chromosomal rearrangements. Such assays may include, but are not limited to, Southern analyses, single stranded conformational polymorphism analyses (SSCP), and PCR analyses.

Such diagnostic methods for the detection of PIKAP specific mutations can involve for example, contacting and incubating nucleic acids including
20 recombinant DNA molecules, cloned genes or degenerate variants thereof, obtained from a sample, e.g., derived from a patient sample or other appropriate cellular source, with one or more labeled nucleic acid reagents including recombinant DNA molecules, cloned genes or degenerate variants thereof, as described above, under conditions favorable for the specific annealing of these
25 reagents to their complementary sequences within the PIKAP. Preferably, the lengths of these nucleic acid reagents are at least 15 to 30 nucleotides. After incubation, all non-annealed nucleic acids are removed from the nucleic acid molecule hybrid. The presence of nucleic acids which have hybridized, if any such molecules exist, is then detected. Using such a detection scheme, the
30 nucleic acid from the cell type or tissue of interest can be immobilized, for example, to a solid support such as a membrane, or a plastic surface such as that on a microtiter plate or polystyrene beads. In this case, after incubation, non-annealed, labeled nucleic acid reagents of the type described above are easily removed. Detection of the remaining, annealed, labeled PIKAP nucleic
35 acid reagents is accomplished using standard techniques well known to those in the art. The PIKAP gene sequences to which the nucleic acid reagents have annealed can be compared to the annealing pattern expected from a normal gene sequence in order to determine whether a gene mutation is present.

Alternative diagnostic methods for the detection of PIKAP gene specific nucleic acid molecules, in patient samples or other appropriate cell sources, may involve their amplification, e.g., by PCR (the experimental embodiment set forth in Mullis, K.B., 1987, U.S. Patent No. 4,683,202), followed by the detection of the amplified molecules using techniques well known to those of skill in the art. The resulting amplified sequences can be compared to those which would be expected if the nucleic acid being amplified contained only normal copies of the PIKAP gene in order to determine whether a gene mutation exists.

Additionally, well-known genotyping techniques can be performed to identify individuals carrying PIKAP gene mutations. Such techniques include, for example, the use of restriction fragment length polymorphisms (RFLPs), which involve sequence variations in one of the recognition sites for the specific restriction enzyme used.

The level of PIKAP gene expression can also be assayed by detecting and measuring PIKAP transcription. For example, RNA from a cell type or tissue known, or suspected to express the PIKAP gene, such as hematopoietic lineage cells, especially myeloid cells and platelets, may be isolated and tested utilizing hybridization or PCR techniques such as are described, above. The isolated cells can be derived from cell culture or from a patient. The analysis of cells taken from culture may be a necessary step in the assessment of cells to be used as part of a cell-based gene therapy technique or, alternatively, to test the effect of compounds on the expression of the PIKAP gene. Such analyses may reveal both quantitative and qualitative aspects of the expression pattern of the PIKAP, including activation or inactivation of PIKAP gene expression.

DETECTION OF THE PIKAP GENE PRODUCTS

Antibodies directed against wild type or mutant PIKAP gene products or conserved variants or peptide fragments thereof, which are discussed, above, may also be used as cell growth disorder diagnostics and prognostics, as described herein. Such diagnostic methods, may be used to detect abnormalities in the level of PIKAP gene expression, or abnormalities in the structure and/or temporal, tissue, cellular, or subcellular location of the PIKAP, and may be performed *in vivo* or *in vitro*, such as, for example, on biopsy tissue.

The tissue or cell type to be analyzed will generally include those which are known, or suspected, to contain cells express the PIKAP gene, such as, for example, neutrophil cells which have infiltrated an inflamed tissue. The protein isolation methods employed herein may, for example, be such as those described in Harlow and Lane (Harlow, E. and Lane, D., 1988, "Antibodies: A Laboratory Manual", Cold Spring Harbor Laboratory Press, Cold Spring Harbor,

New York), which is incorporated herein by reference in its entirety. The isolated cells can be derived from cell culture or from a patient. The analysis of cells taken from culture may be a necessary step in the assessment of cells that could be used as part of a cell-based gene therapy technique or, alternatively, to test the effect of compounds on the expression of the PIKAP gene.

For example, antibodies, or fragments of antibodies, such as those described above are useful in the present invention to quantitatively or qualitatively detect the presence of PIKAP gene products or conserved variants or peptide fragments thereof. This can be accomplished, for example, by immunofluorescence techniques employing a fluorescently labeled antibody (see below) coupled with light microscopic, flow cytometric, or fluorimetric detection.

The antibodies (or fragments thereof) or fusion or conjugated proteins useful in the present invention may, additionally, be employed histologically, as in immunofluorescence, immunoelectron microscopy or non-immuno assays, for *in situ* detection of PIKAP gene products or conserved variants or peptide fragments thereof, or for p85 binding (in the case of labeled p85 fusion protein).

In situ detection may be accomplished by removing a histological specimen from a patient, and applying thereto a labeled antibody or fusion protein of the present invention. The antibody (or fragment) or fusion protein is preferably applied by overlaying the labeled antibody (or fragment) onto a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of the PIKAP gene product, or conserved variants or peptide fragments, but also its distribution in the examined tissue. Using the present invention, those of ordinary skill will readily perceive that any of a wide variety of histological methods (such as staining procedures) can be modified in order to achieve such *in situ* detection.

Immunoassays and non-immunoassays for PIKAP gene products or conserved variants or peptide fragments thereof will typically comprise incubating a sample, such as a biological fluid, a tissue extract, freshly harvested cells, or lysates of cells which have been incubated in cell culture, in the presence of a detectably labeled antibody capable of identifying PIKAP gene products or conserved variants or peptide fragments thereof, and detecting the bound antibody by any of a number of techniques well-known in the art.

The biological sample may be brought in contact with and immobilized onto a solid phase support or carrier such as nitrocellulose, or other solid support which is capable of immobilizing cells, cell particles or soluble proteins. The support may then be washed with suitable buffers followed by treatment with the detectably labeled PIKAP antibody or fusion protein. The solid phase support may then be washed with the buffer a second time to remove unbound antibody

or fusion protein. The amount of bound label on solid support may then be detected by conventional means.

"Solid phase support or carrier" is intended to encompass any support capable of binding an antigen or an antibody. Well-known supports or carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, gabbros, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to an antigen or antibody. Thus, the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. Preferred supports include polystyrene beads. Those skilled in the art will know many other suitable carriers for binding antibody or antigen, or will be able to ascertain the same by use of routine experimentation.

The binding activity of a given lot of PIKAP subunit antibody or fusion protein may be determined according to well known methods. Those skilled in the art will be able to determine operative and optimal assay conditions for each determination by employing routine experimentation.

With respect to antibodies, one of the ways in which the antibody can be detectably labeled is by linking the same to an enzyme and use in an enzyme immunoassay (EIA) (Voller, "The Enzyme Linked Immunosorbent Assay (ELISA)", 1978, Diagnostic Horizons 2:1-7, Microbiological Associates Quarterly Publication, Walkersville, MD); Voller et al., 1978, J. Clin. Pathol. 31:507-520; Butler, 1981, Meth. Enzymol. 73:482-523; Maggio (ed.), 1980, Enzyme Immunoassay, CRC Press, Boca Raton, FL.; Ishikawa et al., (eds.), 1981, Enzyme Immunoassay, Kigaku Shoin, Tokyo). The enzyme which is bound to the antibody will react with an appropriate substrate, preferably a chromogenic substrate, in such a manner as to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorimetric or by visual means. Enzymes which can be used to detectably label the antibody include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate, dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase. The detection can be accomplished by colorimetric methods which employ a chromogenic substrate for the enzyme. Detection may

also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

Detection may also be accomplished using any of a variety of other immunoassays. For example, by radioactively labeling the antibodies or antibody fragments, it is possible to detect PIKAP through the use of a radioimmunoassay (RIA) (see, for example, Weintraub, B., Principles of Radioimmunoassays, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March, 1986, which is incorporated by reference herein). The radioactive isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography.

It is also possible to label the antibody with a fluorescent compound. When the fluorescently labeled antibody is exposed to light of the proper wave length, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin and fluorescamine.

The antibody can also be detectably labeled using fluorescence emitting metals such as ^{152}Eu , or others of the lanthanide series. These metals can be attached to the antibody using such metal chelating groups as diethylenetriaminepentacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

The antibody also can be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound may be used to label the antibody of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in, which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

SCREENING ASSAYS FOR COMPOUNDS THAT MODULATE PI3K EXPRESSION OR ACTIVITY

The following assays are designed to identify compounds that interact with (e.g., bind to) PIKAP or p85, to affect the binding of PIKAP to p85, compounds

that interact with (e.g., bind to) intracellular proteins that interact with PIKAP and/or p85, compounds that interfere with the interaction of PIKAP with p85 or with other intracellular proteins involved in PI3K mediated signal transduction, and to compounds which modulate the activity of PIKAP gene (i.e., modulate the level of PIKAP gene expression) or modulate the level of PIKAP. Assays may additionally be utilized which identify compounds which bind to PIKAP gene regulatory sequences (e.g., promoter sequences) and which may modulate PIKAP gene expression. See e.g., Platt, K.A., 1994, J. Biol. Chem. 269:28558-28562, which is incorporated herein by reference in its entirety.

The compounds which may be screened in accordance with the invention include but are not limited to peptides, antibodies and fragments thereof, prostaglandins, lipids and other organic compounds (e.g., terpenes, peptidomimetics) that bind to PIKAP or p85 and either mimic the activity triggered by the natural ligand (i.e., agonists) or inhibit the activity triggered by the natural ligand (i.e., antagonists); as well as peptides, antibodies or fragments thereof, and other organic compounds that mimic PIKAP or p85 (or a portion thereof) and bind to and "neutralize" natural ligand.

Such compounds may include, but are not limited to, peptides such as, for example, soluble peptides, including but not limited to members of random peptide libraries (see, e.g., Lam, K.S. et al., 1991, Nature 354:82-84; Houghten, R. et al., 1991, Nature 354:84-86), and combinatorial chemistry-derived molecular library peptides made of D- and/or L- configuration amino acids, phosphopeptides (including, but not limited to members of random or partially degenerate, directed phosphopeptide libraries; see, e.g., Songyang, Z. et al., 1993, Cell 72:767-778); antibodies (including, but not limited to, polyclonal, monoclonal, humanized, anti-idiotypic, chimeric or single chain antibodies, and FAb, F(ab.)₂ and FAb expression library fragments, and epitope-binding fragments thereof); and small organic or inorganic molecules.

Other compounds which can be screened in accordance with the invention include but are not limited to small organic molecules that are able to gain entry into an appropriate cell (e.g., in the neutrophil) and affect the expression of the PIKAP gene or some other gene involved in the PIKAP signal transduction pathway (e.g., by interacting with the regulatory region or transcription factors involved in gene expression); or such compounds that affect the activity of the PIKAP, e.g., by inhibiting or enhancing the binding of PIKAP to p85 of PI3K or the binding of PIKAP to some other intracellular factor involved in the PIKAP signal transduction pathway.

Computer modeling and searching technologies permit identification of compounds, or the improvement of already identified compounds, that can modulate PIKAP or p85 expression or activity. Having identified such a compound or composition, the active sites or regions are identified. Such active sites might typically be the binding partner sites, such as, for example, the interaction domains of the p85 with PIKAP itself. Keep in mind that it is shown in the instant patent application that PIKAP and p85 bind to p110 at the same region. The active site can be identified using methods known in the art including, for example, from the amino acid sequences of peptides, from the nucleotide sequences of nucleic acids, or from study of complexes of the relevant compound or composition with its natural ligand. In the latter case, chemical or X-ray crystallographic methods can be used to find the active site by finding where on the factor the complexed ligand is found.

Next, the three dimensional geometric structure of the active site is determined. This can be done by known methods, including X-ray crystallography, which can determine a complete molecular structure. On the other hand, solid or liquid phase NMR can be used to determine certain intramolecular distances. Any other experimental method of structure determination can be used to obtain partial or complete geometric structures. The geometric structures may be measured with a complexed ligand, natural or artificial, which may increase the accuracy of the active site structure determined.

If an incomplete or insufficiently accurate structure is determined, the methods of computer based numerical modeling can be used to complete the structure or improve its accuracy. Any recognized modeling method may be used, including parameterized models specific to particular biopolymers such as proteins or nucleic acids, molecular dynamics models based on computing molecular motions, statistical mechanics models based on thermal ensembles, or combined models. For most types of models, standard molecular force fields, representing the forces between constituent atoms and groups, are necessary, and can be selected from force fields known in physical chemistry. The incomplete or less accurate experimental structures can serve as constraints on the complete and more accurate structures computed by these modeling methods.

Finally, having determined the structure of the active site, either experimentally, by modeling, or by a combination, candidate modulating compounds can be identified by searching databases containing compounds along with information on their molecular structure. Such a search seeks compounds having structures that match the determined active site structure and that interact with the groups defining the active site. Such a search can be

manual, but is preferably computer assisted. These compounds found from this search are potential PIKAP modulating compounds.

Alternatively, these methods can be used to identify improved modulating compounds from an already known modulating compound or ligand. The composition of the known compound can be modified and the structural effects of modification can be determined using the experimental and computer modeling methods described above applied to the new composition. The altered structure is then compared to the active site structure of the compound to determine if an improved fit or interaction results. In this manner systematic variations in composition, such as by varying side groups, can be quickly evaluated to obtain modified modulating compounds or ligands of improved specificity or activity.

Further experimental and computer modeling methods useful to identify modulating compounds based upon identification of the active sites of PIKAP that interact with p85, and related transduction and transcription factors will be apparent to those of skill in the art.

Examples of molecular modeling systems are the CHARMM and QUANTA programs (Polygen Corporation, Waltham, MA). CHARMM performs the energy minimization and molecular dynamics functions. QUANTA performs the construction, graphic modeling and analysis of molecular structure. QUANTA allows interactive construction, modification, visualization, and analysis of the behavior of molecules with each other.

A number of articles review computer modeling of drugs interactive with specific proteins, such as Rotivinen et al., 1988, Acta Pharmaceutica Fennica 97:159-166; Ripka, New Scientist 54-57 (June 16, 1988); McKinaly and Rossmann, 1989, Annu. Rev. Pharmacol. Toxicol. 29:111-122; Perry and Davies, OSAR: Quantitative Structure-Activity Relationships in Drug Design pp.-189-193 (Alan R. Liss, Inc. 1989); Lewis and Dean, 1989, Proc. R. Soc. Lond. 236:125-140 and 141-162; and, with respect to a model receptor for nucleic acid components, Askew et al., 1989, J. Am. Chem. Soc. 111:1082-1090. Other computer programs that screen and graphically depict chemicals are available from companies such as BioDesign, Inc. (Pasadena, CA.), Allelix, Inc. (Mississauga, Ontario, Canada), and Hypercube, Inc. (Cambridge, Ontario). Although these are primarily designed for application to drugs specific to particular proteins, they can be adapted to design of drugs specific to regions of DNA or RNA, once that region is identified.

Although described above with reference to design and generation of compounds which could alter binding, one could also screen libraries of known compounds, including natural products or synthetic chemicals, and biologically

active materials, including proteins, for compounds which are inhibitors or activators.

Compounds identified via assays such as those described herein may be useful, for example, in elaborating the biological function of the PIKAP gene product, and for ameliorating cell growth disorders. Assays for testing the effectiveness of compounds, identified by techniques described herein are discussed below.

In vitro SCREENING ASSAYS FOR COMPOUNDS THAT BIND TO PIKAP

In vitro systems may be designed to identify compounds capable of interacting with (e.g., binding to) PIKAP and p85. Compounds identified may be useful, for example, in modulating the activity of wild type and/or mutant PIKAP gene products; may be utilized in screens for identifying compounds that disrupt normal PIKAP/p85 interactions; or may in themselves disrupt such interactions.

The principle of the assays used to identify compounds that bind to the PIKAP involves preparing a reaction mixture of the PIKAP and the test compound under conditions and for a time sufficient to allow the two components to interact and bind, thus forming a complex which can be removed and/or detected in the reaction mixture. The PIKAP species used can vary depending upon the goal of the screening assay. For example, where agonists of the natural ligand are sought, the full length PIKAP, or a fusion protein containing the PIKAP fused to a protein or polypeptide that affords advantages in the assay system (e.g., labeling, isolation of the resulting complex, etc.) can be utilized.

The screening assays can be conducted in a variety of ways. For example, one method to conduct such an assay would involve anchoring the PIKAP protein, polypeptide, peptide or fusion protein or the test substance onto a solid phase and detecting PIKAP/test compound complexes anchored on the solid phase at the end of the reaction. In one embodiment of such a method, the PIKAP reactant may be anchored onto a solid surface, and the test compound, which is not anchored, may be labeled, either directly or indirectly. In another embodiment of the method, a PIKAP protein anchored on the solid phase is complexed with labeled p85. Then, a test compound could be assayed for its ability to disrupt the association of the PIKAP/p85 complex.

In practice, microtiter plates may conveniently be utilized as the solid phase. The anchored component may be immobilized by non-covalent or covalent attachments. Non-covalent attachment may be accomplished by simply coating the solid surface with a solution of the protein and drying. Alternatively, an immobilized antibody, preferably a monoclonal antibody, specific for the protein

to be immobilized may be used to anchor the protein to the solid surface. The surfaces may be prepared in advance and stored.

In order to conduct the assay, the nonimmobilized component is added to the coated surface containing the anchored component. After the reaction is complete, unreacted components are removed (e.g., by washing) under conditions such that any complexes formed will remain immobilized on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Where the previously nonimmobilized component is pre-labeled, the detection of label immobilized on the surface indicates that complexes were formed. Where the previously nonimmobilized component is not pre-labeled, an indirect label can be used to detect complexes anchored on the surface; e.g., using a labeled antibody specific for the previously nonimmobilized component (the antibody, in turn, may be directly labeled or indirectly labeled with a labeled anti-Ig antibody).

Alternatively, a reaction can be conducted in a liquid phase, the reaction products separated from unreacted components, and complexes detected; e.g., using an immobilized antibody specific for PIKAP protein, polypeptide, peptide or fusion protein, or the p85 protein or fusion protein, or the test compound to anchor any complexes formed in solution, and a labeled antibody specific for the other component of the possible complex to detect anchored complexes.

ASSAYS FOR INTRACELLULAR PROTEINS THAT INTERACT WITH PIKAP

Methods suitable for detecting protein-protein interactions may be employed for identifying intracellular proteins that interact with PIKAP and/or p85. These methods may also be applied to determine the site(s) of interaction of PIKAP with p85. Among the traditional methods which may be employed are co-immunoprecipitation, crosslinking and co-purification through gradients or chromatographic columns of cell lysates or proteins obtained from cell lysates and the PIKAP to identify proteins in the lysate that interact with the PIKAP. For these assays, the PIKAP component used can be a full length PIKAP, or a truncated peptide. Similarly, the component may be p85 and PIKAP, or a complex of PIKAP with p110 and p85. Once isolated, such an intracellular protein can be identified and can, in turn, be used, in conjunction with standard techniques, to identify proteins with which it interacts. The amino acid sequence of an intracellular protein which interacts with the PIKAP can be ascertained using techniques well known to those of skill in the art, such as via the Edman degradation technique. (See, e.g., Creighton, 1983, "Proteins: Structures and Molecular Principles", W.H. Freeman & Co., N.Y., pp.34-49). The amino acid

sequence obtained may be used as a guide for the generation of oligonucleotide mixtures that can be used to screen for gene sequences encoding such intracellular proteins. Screening may be accomplished, for example, by standard hybridization or PCR techniques. Techniques for the generation of

- 5 oligonucleotide mixtures and the screening are well-known. (See, e.g., Ausubel, supra., and PCR Protocols: A Guide to Methods and Applications, 1990, Innis, M. et al., eds. Academic Press, Inc., New York).

- Additionally, methods may be employed which result in the simultaneous identification of genes which encode the intracellular proteins interacting with
10 PIKAP. These methods include, for example, probing expression, libraries, in a manner similar to the well known technique of antibody probing of gt11 libraries, using labeled PIKAP protein, or a PIKAP polypeptide, peptide or fusion protein, e.g., a PIKAP polypeptide or PIKAP domain fused to a marker (e.g., an enzyme, fluor, luminescent protein, or dye), or an Ig-Fc domain.

- 15 One method which detects protein interactions *in vivo*, the two-hybrid system, is described above in detail for illustration only and not by way of limitation. One version of this system has been described (Chien et al., 1991, Proc. Natl. Acad. Sci. USA, 88:9578-9582) and is commercially available from Clontech (Palo Alto, CA). Using this method with specific PIKAP and p85 constructs it was shown
20 that PIKAP binds to p85 at the intermediate SH2 domain. The following constructs were generated and tested in the two-hybrid assay. Table 1 shows the construction strategy for the various p85 or PIKAP constructs that were tested.

Table 1
Plasmid Constructions

- p85 full length: Asp 718 (bp56) filled in with Klenow -PstI (bp2315) of human p85 α into SmaI-PstI of the GBT8 vector.
- 5 p85 SH3 domain: Asp 178 (bp56) filled in with Klenow - XhoII (bp390) of human p85 α into SmaI-BamHI of the GBT8 vector.
- p85 Bcr homology domain: XhoII (bp 390) filled in with Klenow - XhoI (bp 1055) of human p85 α into BamHI filled in with Klenow - Sall of the GBT8 vector.
- 10 p85 SH2 domains and inter SH2 domain: Scal (bp 830) - PstI (bp2315) of human p85 α into SmaI-PstI of the GBT8 vector.
- p85 N-terminal SH2 and inter SH2 domain: p85 SH2 domains and inter SH2 domain construct cut with RcaI (bp1896) and StuI (bp2263), filled in with Klenow and religated without this RcaI-StuI fragment.
- p85 C-terminal SH2 domain: RcaI (bp 1896) filled in with Klenow - StuI(bp2263) of human p85 α into BamHI filled in with Klenow of the GBT8 vector.
- 15 p85 inter SH2 domain:DpnI (bp1343) - RcaI (bp1896) filled in with Klenow of the human p85 α into SmaI of the GBT8 vector.
- PIKAP AA 277-589: original isolate from two hybrid screening: EcoRI linker (5'GAATTCGGGCACGAG3') fused to bp989 of submitted PIKAP sequence to bp 2219 in submitted sequence fused to XhoI linker into EcoRI - XhoI of the GADGH vector.
- 20 PIKAP AA 277-360 : EcoRI linker fused to bp989 (see above) -BamHI (bp1232) into EcoRI - BamHI of the GADGH vector.
- PIKAP AA 361-516: BamHI (bp1232) -Scal (bp1703) into BamHI - SmaI of the GADGH vector.
- PIKAP AA517-589: Scal (bp 1703) - EcoRV (bp1974) into ClaI filled in with Klenow of the GADGH vector.
- 25

Table 2 shows the results of performing the two-hybrid assay using the above constructs. It is apparent that the three constructs that have the intermediate SH2 domain deleted do not bind any of the PIKAP constructs. That is: p85 constructs containing only the sh3 domain, aa 6-117, or the Bcr homology domain, aa 118-340, or the c-terminal sh2 domain, do not exhibit binding to any of the PIKAP constructs. In comparison, p85 constructs that contain the inter sh2 domains, including p85 full length (aa 6-727), or sh2 and inter sh2 domains (aa 263-727) or N-terminal sh2 and inter sh2 domains (aa 263-620), or just the inter sh2 domain (aa 435-620), do bind to PIKAP but to only those PIKAP constructs that display the last 74 amino acids of the c-terminal end of the molecule.

30

35

It is note worthy that PIKAP binds to the inter-SH2 region of p85 as does p110.

40 See Klippel et al., Mol. Cell. Biol., 14: pages 2675-2685 (1994). Thus, PIKAP

may have a regulatory role on the catalytic activity of p110 by competing for binding with p85 to the inter sh2 domain on p85.

Table 2
Binding of PIKAP to Inter SH2 of p85

	PIKAP <u>AA 277-589</u>	PIKAP <u>AA 277-360</u>	PIKAP <u>AA 361-516</u>	PIKAP <u>AA 517-</u>
589				
10 p85 full length (AA 6-727)	+++	-	-	+++
p85 SH3 domain (AA 6-117)	-			
15 p85 Bcr homology domain (AA 118-340)	-			
p85 SH2 domains and inter SH2 domain (AA 263-727)	+++	-	-	+++
20 p85 N-terminal SH2 and inter SH2 domain (AA 263-620)	+++	-	-	+++
p85 C-terminal SH2 domain (AA 620-727)	-			
25 p85 inter SH2 domain (AA 435-620)	+++	-	-	+++

RESTORATION OR INCREASE IN PIKAP EXPRESSION OR
ACTIVITY TO PROMOTE CONTROL OF CELL GROWTH

- 30 With respect to an increase in the level of normal PIKAP gene expression and/or PIKAP gene product activity, PIKAP nucleic acid sequences can be utilized for the treatment of cell growth disorders, including restinosis and cancer. Where the cause of the cell growth disorders is a defective PIKAP, treatment can be administered, for example, in the form of gene replacement therapy.
- 35 Specifically, one or more copies of a normal PIKAP gene or a portion of the PIKAP gene that directs the production of a PIKAP gene product exhibiting normal function, may be inserted into the appropriate cells within a patient or animal subject, using vectors which include, but are not limited to adenovirus, adeno-associated virus, retrovirus and herpes virus vectors, in addition to other
- 40 particles that introduce DNA into cells, such as liposomes.

Because the PIKAP gene is expressed in most cells such gene replacement therapy techniques should be capable of delivering PIKAP gene sequences to

these cell types within patients. Alternatively, targeted homologous recombination can be utilized to correct the defective endogenous PIKAP gene in the appropriate cell type; e.g., bone marrow cells or neutrophils and/or other leukocytes.

5 Finally, compounds identified in the assays described above that stimulate, enhance, or modify the signal transduced by activated PIKAP, e.g., by activating downstream signaling proteins in the PIKAP cascade and thereby by-passing the defective PIKAP, can be used to achieve immune system stimulation. The formulation and mode of administration will depend upon the physico-chemical
10 properties of the compound.

PHARMACEUTICAL PREPARATIONS

The compounds that are determined to affect PIKAP gene expression or PIKAP activity, or the interaction of PIKAP with any of its binding partners
15 including but not limited to p85, can be administered to a patient at therapeutically effective doses to treat or ameliorate hematopoietic cell growth disorders, including restinosis, and cancer. A therapeutically effective dose refers to that amount of the compound sufficient to result in amelioration of symptoms of such disorders.

20

EFFECTIVE DOSE

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD₅₀ (the dose lethal to 50% of the population) and the
25 ED₅₀ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD₅₀/ED₅₀. Compounds which exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such
30 compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that
35 include the ED₅₀ with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays.

A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine
5 useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

FORMULATIONS AND USE

Pharmaceutical compositions for use in accordance with the present
10 invention may be formulated in conventional manner using one or more physiologically acceptable carriers or excipients.

Thus, the compounds and their physiologically acceptable salts and solvates may be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral or rectal administration.

15 For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium
20 hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., potato starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulfate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry
25 product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g., sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., almond oil, oily esters, ethyl
30 alcohol or fractionated vegetable oils); and preservatives (e.g., methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

Preparations for oral administration may be suitably formulated to give controlled release of the active compound.

35 For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray

presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g. gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

The following examples are presented to illustrate the present invention and to assist one of ordinary skill in making and using the same. The examples are not intended in any way to otherwise limit the scope of the disclosure or the protection granted by Letters patent hereon.

DEPOSIT OF CLONES

The following microorganisms or clones were deposited with the American Type Culture Collection (ATCC), Rockville, Maryland, on the dates indicated and were assigned the indicated accession number:

Clone
PIKAP

Access. No.
98189

Date of Deposit
September 30, 1996

5 The present invention is not to be limited in scope by the specific
embodiments described herein, which are intended as single illustrations of
individual aspects of the invention, and functionally equivalent methods and
components are within the scope of the invention. Indeed, various modifications
of the invention, in addition to those shown and described herein will become
apparent to those skilled in the art from the foregoing description and
10 accompanying drawings. Such modifications are intended to fall within the scope
of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- 5 (i) APPLICANT: Braselmann, Sylvia
- (ii) TITLE OF INVENTION: Nucleotide Sequences that Encode
Phosphatidylinositol-3' Kinase Associated Proteins and
Uses Thereof
- 10 (iii) NUMBER OF SEQUENCES: 2
- (iv) CORRESPONDENCE ADDRESS:
- (A) ADDRESSEE: ONYX Pharmaceuticals, Inc.
- (B) STREET: 3031 Research Drive
- (C) CITY: Richmond
- (D) STATE: CA
- 15 (E) COUNTRY: USA
- (F) ZIP: 94806
- (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- 20 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER: US Unknown
- (B) FILING DATE:
- 25 (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
- (A) NAME: Giotta, Gregory
- (B) REGISTRATION NUMBER: 32,028
- (C) REFERENCE/DOCKET NUMBER: ONYX1027
- 30 (ix) TELECOMMUNICATION INFORMATION:
- (A) TELEPHONE: (510) 262-8710
- (B) TELEFAX: (510) 222-9758

(2) INFORMATION FOR SEQ ID NO:1:

- 35 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 2307 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear
- 40 (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (ix) FEATURE:
- (A) NAME/KEY: CDS
- 45 (B) LOCATION: 162..1928
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTCGAGGGGC ATCGCGCCGC CCGGCGCGCG CCGCCCCCT GCCTCGCGGC GCGGGGTCTC

60

GCGGGCCCCG CTCCCGCCCT CCGCTCGCCT GGCCCGGACC GGAAGCGGCG CCGCACGGCC 120
TGGGCCTGGC GCGGGGGGCG GGCACCGGGG CCCGGTCGGA C ATG GGC AAG AAG 173
Met Gly Lys Lys
1
5 CAC AAG AAG CAC AAG TCG GAC AAA CAC CTC TAC GAG GAG TAT GTA GAG 221
His Lys Lys His Lys Ser Asp Lys His Leu Tyr Glu Glu Tyr Val Glu
5 10 15 20
AAG CCC TTG AAG CTG GTC CTC AAA GTA GGA GGG AAC GAA GTC ACC GAA 269
Lys Pro Leu Lys Leu Val Leu Lys Val Gly Gly Asn Glu Val Thr Glu
10 25 30 35
CTC TCC ACG GGC AGC TCG GGG CAC GAC TCC AGC CTC TTC GAA GAC AAA 317
Leu Ser Thr Gly Ser Ser Gly His Asp Ser Ser Leu Phe Glu Asp Lys
40 45 50
AAC GAT CAT GAC AAA CAC AAG GAC AGA AAG CGG AAA AAG AGA AAG AAA 365
Asn Asp His Asp Lys His Lys Asp Arg Lys Arg Lys Lys Arg Lys Lys
55 60 65
GGA GAG AAG CAG ATT CCA GGG GAA GAA AAG GGG AGA AAA CGG AGA AGA 413
Gly Glu Lys Gln Ile Pro Gly Glu Glu Lys Gly Arg Lys Arg Arg Arg
70 75 80
20 GTT AAG GAG GAT AAA AAG AAG CGA GAT CGA GAC CGG GTG GAG AAT GAG 461
Val Lys Glu Asp Lys Lys Lys Arg Asp Arg Asp Arg Val Glu Asn Glu
85 90 95 100
GCA GAA AAA GAT CTC CAG TGT CAC GCC CCT GTG AGA TTA GAC TTG CCT 509
Ala Glu Lys Asp Leu Gln Cys His Ala Pro Val Arg Leu Asp Leu Pro
105 110 115
25 CCT GAG AAG CCT CTC ACA AGC TCT TTA GCC AAA CAA GAA GAA GTA GAA 557
Pro Glu Lys Pro Leu Thr Ser Ser Leu Ala Lys Gln Glu Glu Val Glu
120 125 130
30 CAG ACA CCC CTT CAA GAA GCT TTG AAT CAA CTG ATG AGA CAA TTG CAG 605
Gln Thr Pro Leu Gln Glu Ala Leu Asn Gln Leu Met Arg Gln Leu Gln
135 140 145
AGA AAA GAT CCA AGT GCT TTC TTT TCA TTT CCT GTG ACT GAT TTT ATT 653
Arg Lys Asp Pro Ser Ala Phe Phe Ser Phe Pro Val Thr Asp Phe Ile
150 155 160
35 GCT CCT GGC TAC TCC ATG ATC ATT AAA CAC CCA ATG GAT TTT AGT ACC 701
Ala Pro Gly Tyr Ser Met Ile Ile Lys His Pro Met Asp Phe Ser Thr
165 170 175 180
ATG AAA GAA AAG ATC AAG AAC AAT GAC TAT CAG TCC ATA GAA GAA CTA 749
Met Lys Glu Lys Ile Lys Asn Asn Asp Tyr Gln Ser Ile Glu Glu Leu
185 190 195
40 AAG GAT AAC TTC AAA CTA ATG TGT ACT AAT GCC ATG ATT TAC AAT AAA 797
Lys Asp Asn Phe Lys Leu Met Cys Thr Asn Ala Met Ile Tyr Asn Lys
200 205 210
45 CCA GAG ACC ATT TAT TAT AAA GCT GCA AAG AAG CTG TTG CAC TCA GGA 845
Pro Glu Thr Ile Tyr Tyr Lys Ala Ala Lys Lys Leu Leu His Ser Gly
215 220 225
ATG AAA ATT CTT AGC CAG GAA AGA ATT CAG AGC CTG AAG CAG AGC ATA 893
Met Lys Ile Leu Ser Gln Glu Arg Ile Gln Ser Leu Lys Gln Ser Ile

	230	235	240	
	GAC TTC ATG GCT GAC TTG CAG AAA ACT CGA AAG CAG AAA GAT GGA ACA			941
	Asp Phe Met Ala Asp Leu Gln Lys Thr Arg Lys Gln Lys Asp Gly Thr			
	245	250	255	260
5	GAC ACC TCA CAG AGT GGG GAG GAC GGA GGC TGC TGG CAG AGA GAG AGA			989
	Asp Thr Ser Gln Ser Gly Glu Asp Gly Gly Cys Trp Gln Arg Glu Arg			
	265	270	275	
	GAG GAC TCT GGA GAT GCC GAA GCA CAC GCC TTC AAG AGT CCC AGC AAA			1037
	Glu Asp Ser Gly Asp Ala Glu Ala His Ala Phe Lys Ser Pro Ser Lys			
10	280	285	290	
	GAA AAT AAA AAG AAA GAC AAA GAT ATG CTT GAA GAT AAG TTT AAA AGC			1085
	Glu Asn Lys Lys Lys Asp Lys Asp Met Leu Glu Asp Lys Phe Lys Ser			
	295	300	305	
	AAT AAT TTA GAG AGA GAG CAG GAG CAG CTT GAC CGC ATC GTG AAG GAA			1133
15	Asn Asn Leu Glu Arg Glu Gln Glu Gln Leu Asp Arg Ile Val Lys Glu			
	310	315	320	
	TCT GGA GGA AAG CTG ACC AGG CGG CTT GTG AAC AGT CAG TGC GAA TTT			1181
	Ser Gly Gly Lys Leu Thr Arg Arg Leu Val Asn Ser Gln Cys Glu Phe			
	325	330	335	340
20	GAA AGA AGA AAA CCA GAT GGA ACA ACG ACG TTG GGA CTT CTC CAT CCT			1229
	Glu Arg Arg Lys Pro Asp Gly Thr Thr Thr Leu Gly Leu Leu His Pro			
	345	350	355	
	GTG GAT CCC ATT GTA GGA GAG CCA GGC TAC TGC CTG GTG AGA CTG GGA			1277
	Val Asp Pro Ile Val Gly Glu Pro Gly Tyr Cys Leu Val Arg Leu Gly			
25	360	365	370	
	ATG ACA ACT GGA AGA CTT CAG TCT GGA GTG AAT ACT TTG CAG GGG TTC			1325
	Met Thr Thr Gly Arg Leu Gln Ser Gly Val Asn Thr Leu Gln Gly Phe			
	375	380	385	
	AAA GAG GAT AAA AGG AAC AAA GTC ACT CCA GTG TTA TAT TTG AAT TAT			1373
30	Lys Glu Asp Lys Arg Asn Lys Val Thr Pro Val Leu Tyr Leu Asn Tyr			
	390	395	400	
	GGG CCC TAC AGT TCT TAT GCA CCG CAT TAT GAC TCC ACA TTT GCA AAT			1421
	Gly Pro Tyr Ser Ser Tyr Ala Pro His Tyr Asp Ser Thr Phe Ala Asn			
	405	410	415	420
35	ATC AGC AAG GAT GAT TCT GAT TTA ATC TAT TCA ACC TAT GGG GAA GAC			1469
	Ile Ser Lys Asp Asp Ser Asp Leu Ile Tyr Ser Thr Tyr Gly Glu Asp			
	425	430	435	
	TCT GAT CTT CCA AGT GAT TTC AGC ATC CAT GAG TTT TTG GCC ACG TGC			1517
	Ser Asp Leu Pro Ser Asp Phe Ser Ile His Glu Phe Leu Ala Thr Cys			
40	440	445	450	
	CAA GAT TAT CCG TAT GTC ATG GCA GAT AGT TTA CTG GAT GTT TTA ACA			1565
	Gln Asp Tyr Pro Tyr Val Met Ala Asp Ser Leu Leu Asp Val Leu Thr			
	455	460	465	
	AAA GGA GGG CAT TCC AGG ACC CTA CAA GAG ATG GAG ATG TCA TTG CCT			1613
45	Lys Gly Gly His Ser Arg Thr Leu Gln Glu Met Glu Met Ser Leu Pro			
	470	475	480	
	GAA GAT GAA GGC CAT ACT AGG ACA CTT GAC ACA GGA AAA GAA ATG GAG			1661
	Glu Asp Glu Gly His Thr Arg Thr Leu Asp Thr Gly Lys Glu Met Glu			

	485		490		495		500	
	CAG ATT ACA GAA GTA GAG CCA CCA GGG CGT TTG GAC TCC AGT ACT CAA							1709
	Gln Ile Thr Glu Val Glu Pro Pro Gly Arg Leu Asp Ser Ser Thr Gln							
		505		510		515		
5	GAC AGG CTC ATA GCG CTG AAA GCA GTA ACA AAT TTT GGC GTT CCA GTT							1757
	Asp Arg Leu Ile Ala Leu Lys Ala Val Thr Asn Phe Gly Val Pro Val							
		520		525		530		
	GAA GTT TTT GAC TCT GAA GAA GCT GAA ATA TTC CAG AAG AAA CTT GAT							1805
	Glu Val Phe Asp Ser Glu Glu Ala Glu Ile Phe Gln Lys Lys Leu Asp							
10		535		540		545		
	GAG ACC ACC AGA TTG CTC AGG GAA CTC CAG GAA GCC CAG AAT GAA CGT							1853
	Glu Thr Thr Arg Leu Leu Arg Glu Leu Gln Glu Ala Gln Asn Glu Arg							
		550		555		560		
	TTG AGC ACC AGA CCC CCT GGG AAC ATG ATC TGT CTC TTG GGT CCC TCA							1901
15	Leu Ser Thr Arg Pro Pro Gly Asn Met Ile Cys Leu Leu Gly Pro Ser							
		565		570		575		580
	TCA GAG AAA TGC ATC TTG CTG AAC AAG TGACCAATAA TCTTAAAGAA							1948
	Ser Glu Lys Cys Ile Leu Leu Asn Lys							
		585						
20	TTGCACAGCA AGTAACTCCA GGTGATATCG TAAGCACGTA TGGAGTTCGA AAAGCAATGG							2008
	GGATTTCCAT TCCTTCCCCC GTCATGGAAA ACAACTTTGT GGATTGACA GAAGACACTG							2068
	AAGAACCTAA AAAGACGGAT GTTGATGAGT GTCGACCTGG TGGAAGTTGA GGCTGCCTGG							2128
	TATTTGATTA TATATTATGT ACATACTTTT TCATTCTTAA CTTAGAAATG CTTTTCAGAA							2188
	GATATTAAAT ATTTGTAAAT TGTGTTTTTA ATTAACTTT TGGAACAGCG AATTTGGATG							2248
25	TTCCAGAGGT TGGACTTGTA TTAGGTAATA AAGCTGGACC TGGGACTCGT GAGGAAGGA							2307

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 589 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

35	Met Gly Lys Lys His Lys Lys His Lys Ser Asp Lys His Leu Tyr Glu
	1 5 10 15
	Glu Tyr Val Glu Lys Pro Leu Lys Leu Val Leu Lys Val Gly Gly Asn
	20 25 30
	Glu Val Thr Glu Leu Ser Thr Gly Ser Ser Gly His Asp Ser Ser Leu
40	35 40 45
	Phe Glu Asp Lys Asn Asp His Asp Lys His Lys Asp Arg Lys Arg Lys
	50 55 60
	Lys Arg Lys Lys Gly Glu Lys Gln Ile Pro Gly Glu Glu Lys Gly Arg
	65 70 75 80
45	Lys Arg Arg Arg Val Lys Glu Asp Lys Lys Lys Arg Asp Arg Asp Arg
	85 90 95
	Val Glu Asn Glu Ala Glu Lys Asp Leu Gln Cys His Ala Pro Val Arg
	100 105 110

Leu Asp Leu Pro Pro Glu Lys Pro Leu Thr Ser Ser Leu Ala Lys Gln
 115 120 125
 Glu Glu Val Glu Gln Thr Pro Leu Gln Glu Ala Leu Asn Gln Leu Met
 130 135 140
 5 Arg Gln Leu Gln Arg Lys Asp Pro Ser Ala Phe Phe Ser Phe Pro Val
 145 150 155 160
 Thr Asp Phe Ile Ala Pro Gly Tyr Ser Met Ile Ile Lys His Pro Met
 165 170 175
 Asp Phe Ser Thr Met Lys Glu Lys Ile Lys Asn Asn Asp Tyr Gln Ser
 180 185 190
 10 Ile Glu Glu Leu Lys Asp Asn Phe Lys Leu Met Cys Thr Asn Ala Met
 195 200 205
 Ile Tyr Asn Lys Pro Glu Thr Ile Tyr Tyr Lys Ala Ala Lys Lys Leu
 210 215 220
 15 Leu His Ser Gly Met Lys Ile Leu Ser Gln Glu Arg Ile Gln Ser Leu
 225 230 235 240
 Lys Gln Ser Ile Asp Phe Met Ala Asp Leu Gln Lys Thr Arg Lys Gln
 245 250 255
 Lys Asp Gly Thr Asp Thr Ser Gln Ser Gly Glu Asp Gly Gly Cys Trp
 260 265 270
 20 Gln Arg Glu Arg Glu Asp Ser Gly Asp Ala Glu Ala His Ala Phe Lys
 275 280 285
 Ser Pro Ser Lys Glu Asn Lys Lys Lys Asp Lys Asp Met Leu Glu Asp
 290 295 300
 25 Lys Phe Lys Ser Asn Asn Leu Glu Arg Glu Gln Glu Gln Leu Asp Arg
 305 310 315 320
 Ile Val Lys Glu Ser Gly Gly Lys Leu Thr Arg Arg Leu Val Asn Ser
 325 330 335
 Gln Cys Glu Phe Glu Arg Arg Lys Pro Asp Gly Thr Thr Thr Leu Gly
 340 345 350
 30 Leu Leu His Pro Val Asp Pro Ile Val Gly Glu Pro Gly Tyr Cys Leu
 355 360 365
 Val Arg Leu Gly Met Thr Thr Gly Arg Leu Gln Ser Gly Val Asn Thr
 370 375 380
 35 Leu Gln Gly Phe Lys Glu Asp Lys Arg Asn Lys Val Thr Pro Val Leu
 385 390 395 400
 Tyr Leu Asn Tyr Gly Pro Tyr Ser Ser Tyr Ala Pro His Tyr Asp Ser
 405 410 415
 Thr Phe Ala Asn Ile Ser Lys Asp Asp Ser Asp Leu Ile Tyr Ser Thr
 420 425 430
 40 Tyr Gly Glu Asp Ser Asp Leu Pro Ser Asp Phe Ser Ile His Glu Phe
 435 440 445
 Leu Ala Thr Cys Gln Asp Tyr Pro Tyr Val Met Ala Asp Ser Leu Leu
 450 455 460
 45 Asp Val Leu Thr Lys Gly Gly His Ser Arg Thr Leu Gln Glu Met Glu
 465 470 475 480
 Met Ser Leu Pro Glu Asp Glu Gly His Thr Arg Thr Leu Asp Thr Gly
 485 490 495

Lys Glu Met Glu Gln Ile Thr Glu Val Glu Pro Pro Gly Arg Leu Asp
 500 505 510
 Ser Ser Thr Gln Asp Arg Leu Ile Ala Leu Lys Ala Val Thr Asn Phe
 515 520 525
 5 Gly Val Pro Val Glu Val Phe Asp Ser Glu Glu Ala Glu Ile Phe Gln
 530 535 540
 Lys Lys Leu Asp Glu Thr Thr Arg Leu Leu Arg Glu Leu Gln Glu Ala
 545 550 555 560
 Gln Asn Glu Arg Leu Ser Thr Arg Pro Pro Gly Asn Met Ile Cys Leu
 10 565 570 575
 Leu Gly Pro Ser Ser Glu Lys Cys Ile Leu Leu Asn Lys
 580 585